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(71) Applicant *(for all designated States except US)*: THROMBOSIS RESEARCH INSTITUTE [GB/GB]; 90 Fetter Lane, London EC4A 1EQ (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: BLEASDALE, Ciao, Chang [CN/GB]; 45 Oaklands, Westham, Pevensey, Eastbourne BN24 5AW (GB). DEMOLIOU-MASON, Catherine [GB/GB]; 5 Wellesley Mansion, Edith Villas, London W14 9AH (GB). KAKKAR, Vijay, Vir [IN/GB]; 6 Aspen Copse, Bickley, Kent BR1 2NZ (GB).

(74) Agents: LINN, Samuel, Jonathan et al.; Mewburn Ellis, York House, 23 Kingsway, London WC2B 6HP (GB).

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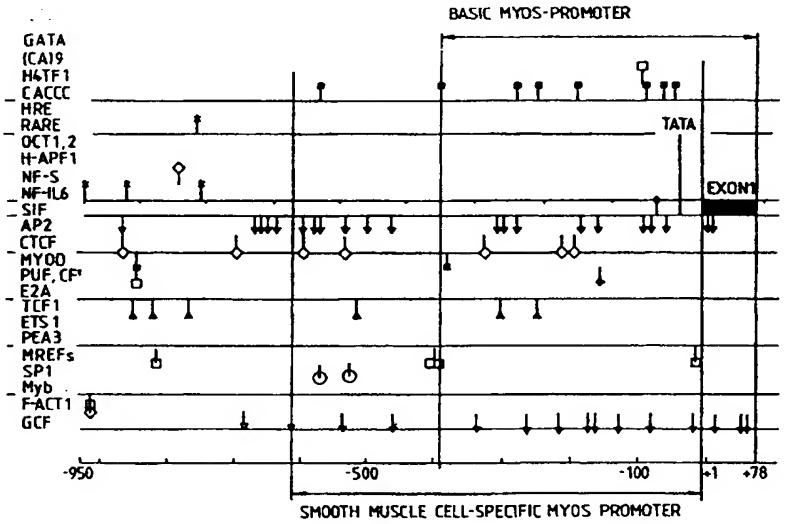

(57) Abstract

The 5' upstream sequence from position -950 to +78 of the rabbit smooth muscle myosin heavy chain gene (Fig. 2) has strong promoter activity in the expression of reporter genes such as CAT and β-gal in a variety of mammalian host cells, e.g. vascular smooth muscle cells. The invention provides methods of such enhanced gene expression, eukaryotic expression vectors therefor, and use of the MYOS promoter in the expression of such genes. The invention has particular applicability to the expression of genes for use in human gene therapy.

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EUKARYOTIC EXPRESSION VECTORS DRIVEN BY A MYOSIN HEAVY CHAIN GENE PROMOTER

FIELD OF THE INVENTION

This invention relates to Eukaryotic expression vectors comprising novel mammalian cellular promoters for tissue-specific expression or ubiquitous expression of genes such as therapeutic genes in vitro and in vivo, for use for example in human gene therapy.

BACKGROUND OF THE INVENTION AND PRIOR ART

Background literature references are referred to herein by way of parenthetical numerical citation in the test to the appended bibliography. The disclosures of these references are incorporated herein by reference.

Monumental progress in the area of biotechnology has led to the development of a variety of prokaryotic and eukaryotic systems for the expression of cloned genes. The typical eukaryotic expression system is usually composed of a number of well characterised viral and mammalian elements. The basic requirements are a promoter element to drive the transcription, the coding sequence and the signals required for efficient processing of the transcript, the ribosome binding site

for translation initiation, the replicon regions for stable replication and copy number control in bacteria and eukaryotic cells and the selectable marker such as a gene conferring antibiotic resistance to the host.

In eukaryotes, the process of transcription is mediated by three DNA-dependent RNA polymerases: I, II and III. Each eukaryotic RNA polymerase is specific for transcribing unique sets of genes: RNA polymerase I synthesises ribosomal pre-RNA; RNA polymerase II transcribes protein coding (class II) genes and most small nuclear RNA (sn RNA); and RNA polymerase III synthesizes small RNA species such as tRNA and ribosomal 5S RNA.

The regulatory regions of RNA polymerase II-transcribed genes contain two types of functional element: CORE PROMOTER elements such as the TATA box located approximately 30 nucleotides upstream (-30) of the transcription initiation site (+1); and INITIATOR-TATA-less promoter, which encompasses the transcription initiate site. The CORE PROMOTER can specify the site at which the RNA polymerase II transcription initiation complex is assembled - the mRNA start site - and mediate the action of some upstream activators. UPSTREAM PROMOTER elements and ENHANCER sequences bind activator proteins that increase the rate of transcription

initiation; these elements are usually 100 - 200 bp in length. ENHANCER sequences can act in either orientation over a considerable distance to active transcription from a linked core promoter. UPSTREAM PROMOTER elements, however, have the orientation requirement (1).

Most eukaryotic genes are discontineous with coding sequences (exons) interrupted by intervening non-coding sequences (introns) that can vary in size. The basic transcription initiation factor TFIID binding to the TATA element is the essential first step in the stepwise assembly of the transcription complex. The TATA element interaction is mediated by the TATA-binding protein (TBP), a protein that is highly conserved across eukaryotic species which can function with the other basic RNA polymerase II initiation factors in supporting the transcription. RNA polymerase II initiates transcription at the promoter of the gene, reads through these exons and introns and passes into the 3'-flanking region. The majority of eukaryotic mRNAs are polyadenylated for stability reasons (37). A general strategy to examine the contributions of regulatory elements of the 5'-upstream region of the gene of interest, is to link the regulatory sequences to reporter genes such as the coding sequences for the bacterial chloramphenicol acetyltranstefase enzyme (CAT)(8,32), or β-galactosidase (9,33) or the firefly luciferase gene

(10). The amount of enzymatic activity which accumulates after the transfection is taken as a measure of the ability of the DNA sequences studied to either initiate or to regulate the transcription.

Many viral promoter/enhancer combinations are active in a variety of tissues or cells and therefore have been extensively used for the construction of vectors; for example, those based on the Semian Virus 40 (SV40), Rous Sarcoma Virus (RSV) and Cytomegalovirus (CMV); and those specialised vectors derived from Retroviruses, Adenoviruses, Adeno-associated Virus (AAV), Epstein-Barr Virus (EBV), Herpes Simplex Virus (HSV), Vaccinia Virus, etc. (2-7).

Gene therapy is a medical intervention to alter the genetic program of cells for therapeutic purposes. This innovative approach to therapeutics has the potential to prevent, treat or potentially cure a variety of inherited and acquired diseases. Two general strategies are being developed for the treatment of diseases based on somatic cell gene therapy: Ex vivo gene therapy is a multistep therapy which involves transplantation of genetically modified autologous cells. An advantage of this approach is that gene transfer can be accomplished in an efficient and controlled manner in vitro and the genetically modified cells can be characterised prior to

transplantations. However, it is generally an invasive therapy requiring two surgical procedures. An alternative strategy is direct delivery of the therapeutic gene to cells - in vivo gene therapy - which would require the development of gene transfer systems capable of targeting to the appropriate cell and internalizing the gene so that it is transported to the nucleus and expressed. Successful application of gene therapy for the treatment of human diseases therefore requires the further development of safe and efficient methods of transferring genetic material into somatic cells or tissues (40).

To date, the most commonly used gene transfer system is based on retroviral vectors and adenoviral vectors that shuttle the gene of interest into the recipient cell with great efficiency. However, the possibility of promoter inactivation that occurs in vivo with viral promoters and consideration of safety issues for future large scale clinical trials and targeting gene transfer strategies require the further development of novel gene transfer systems. In particular, the use of strong cellular promoters with tissue specific expression function will make new contributions to this field.

Recombinant vectors can be introduced into cultured mammalian cells by a variety of methods including that of

calcium phosphate precipitation (14), electroporation (15) and liposomes (16) or receptor mediated endocytosis (17,18). For in vivo direct gene transfer, recombinant virus transduction, liposome/DNA and receptor mediated endocytosis have been used successfully. In addition, a number of new techniques are being developed.

Vascular smooth muscle cell (VSMC) migration and proliferation in vivo has been associated with the development of atherosclerosis and in intimal hyperplasia after vessel wall injury. The phenotype of these proliferating VSMC is considered to be similar to that observed in in vivo cultures of VSMC. Proliferative VSMC in vitro undergo phenotypic modulation from the "contractile" to the "synthetic" phenotype which is associated with the loss of myosin thick filaments and contractile ability as well as an increase in subcellular organelles for protein synthesis (20).

Myosin is one of the major contractile proteins in all three types of muscles - skeletal muscle (e.g. arms, legs, spine - fast and slow skeletal muscles), cardiac muscle (e.g. heart - atrial and neutricular cardiac muscles) and smooth muscle (e.g. large blood vessels, intestines and gall bladder - vascular and other smooth muscles) - which posseses both globular enzyme and fibrous structural protein functions. Each hexameric

myosin molecule is composed of two myosin heavy chains (MHCs) and four myosin light chains (MLCs). MHC is a complex multifunctional protein with both enzymatic and structural domains, its globular S1 head has ATPase activity which is essential for the contractile activity, and its a-helical tail forms the coiled-coil dimer as the basic unit to form thick filaments.

The skeletal, cardiac and smooth muscle MHCs are encoded by different gene families: the skeletal MHCs are encoded by a highly conserved multigene family; the α- and β-cardiac MHCs are encoded by two separate genes; (21-23); however, so far all described smooth muscle MHC isoforms (SM1, SM2, SMB) have been shown to be the result of alternative splicing of RNA from a single gene which results in divergences at the carboxyl termini (SM1 & SM2), or at the 25/50 kD junction, adjacent to the ATP binding site (SMB) (24-26).

The expression of smooth MHCs are regulated developmentally and in a tissue-specific manner. Whereas SM1 and SM2 are expressed equally in normal adult muscles, only SM1 is expressed in fetal and perinatal aortas. In the VSMC of arteriosclerotic neointimas in the rabbit, SM1 expression was maintained at a high level and the SM2 expression decreased, suggesting that dedifferentiation of VSMC may be involved in the cellular

mechanism of atherosclerosis (25,26). The tissue-specific and differential expression of the smooth MHC isoforms make the smooth MHC gene a good marker for the study of VSMC differentiation and the cellular mechanisms underlying their abnormal proliferation in atherosclerosis.

To study the tissue-specific and differential expression of the smooth MHC gene and its regulatory mechanisms, we have isolated, cloned and characterised the 5' upstream promoter region of the rabbit smooth MHC gene. We have discovered that the 5' upstream 1028 bp region (-950 to +78) of the rabbit smooth MHC gene contains the core promoter activity and positive and negative regulatory elements which contribute to the expression level or tissue-specificity driven from this novel promoter, which is hereinafter referred to as "MYOS Promoter". This fragment contains a consensus "TATA box" (-30), the transcription initiation site (+1), the untranslated first exon of rabbit smooth MHC gene (+1 - +78), and potential GC rich sequences with multiple binding sites of the transcription factors. This novel cellular promoter can drive transient CAT reporter gene expression in transfected rabbit and human VSMC cells to levels as much as 5- to 10-fold higher than those driven by the SV40 promoter (i.e. pCAT promoter, Promega). In vivo liposome/DNA direct gene deliver system in the

rabbit model showed this MYOS promoter can drive β-gal reporter gene expression in rabbit artery vessel walls in vivo.

The MYOS promoter, containing the -950 to +78 (5' to 3' orientation) sequence and its nested 5' end deletion mutants were used as promoter/enhancer in the construction of a series of novel eukaryotic expression vectors (pTRm1 series) which either contain the MYOS promoter sequence, the EBNA-a/oriP sequence for eukaryotic cell episomal replication, a multi-cloning site for inserting a foreign gene of interest, with a SV-polyadenylation site, a neomycin resistance gene driven by TK promoter as the selection marker in eukaryotic cells, the Ampicillin resistance gene and ColE1 ori sequences for the selection and replication in bacteria strains (pTRm1 series, as shown in Fig. 6 of the accompanying drawings); or contain the MYOS promoter sequences, the multi-clonal site, with bovine growth hormone poly A signal, f1 origin, the neomycin resistance gene driven by SV40 promoter for the expression and selection in eukaryotic cells, also with the E.coli shuttle elements, ColE1 ori and the Ampicillin resistance gene as selection marker (pTRm2 series, as shown in Fig. 7 of the accompanying drawings).

These vectors are designed for high level transient

or stable expression of recombinant therapeutic genes or any coding sequences of interest in vitro in culturing eukaryotic cells, in vivo in animal model studies and for therapeutic purposes in human gene therapy. They can replicate episomally for high copy numbers (pTRm1 vectors) or integrate into chromosomes (pTRm2 vectors). They have a broad range of host cells and can be used for studies on the regulation of gene expression, for the characterization of proteins, for the cloning and testing of new designs of recombinant or artificial therapeutic genes in both basic research and medical application fields. The pTRm1S and pTRm2S vectors have special value in VSMC cell-type specific expression patterns which can be used as such or upon further modification in cardiovascular disease research and in the design of new strategies for cardiovascular gene therapy, e.g. targeting inhibition of proliferating VSMC cells involved in atherosclerosis.

SUMMARY OF THE INVENTION

The promoter activity of the 5' upstream sequence from position -950 to position +78 of the rabbit smooth muscle myosin heavy chain gene, (MYOS promoter) can drive the expression of reporter genes such as the CAT gene or β-gal gene in vitro and in vivo to levels equal to or higher than those obtained with the same reporter genes

driven by SV40 promoter/enhancer in transient expression studies in rabbit and human SMC cells, as well as via the in vivo direct gene transfer system (liposome/DNA) where significant expression was reached in rabbit artery vessel walls in vivo.

The core promoter sequence of the MYOS promoter has been defined as extending to position -180 upstream of the transcription initiation site. It contains a TATA-box and showed the promoter activity in a number of cell types i.e. primary rabbit vascular SMC cells, primary human vascular SMC cells, human dermal fibroblasts (HDF), rabbit skin fibroblast cell line (RAB-9), rabbit kidney epithelial cell line (RK13) and rat skeletal muscle myoblasts cell line (L6).

A proximal 5' upstream negative element (between position -390 to -180), and a distal positive domain (between positions -638 to -504) in the 5' upstream region of the MYOS promoter sequence have been defined, for the regulation of expression activity in rabbit and human vascular SMC cells.

The untranslated first exon sequence (+1 to +78) interacts with the 5' distal positive domain (between -638 and -505) to regulate the universal full activity of the MYOS promoter in those different cell types mentioned

above.

Without the Exon 1 sequence, the MYOS promoter activity up to position -638 was greatly decreased in other cell types (i.e. L6 and HDF cells, more than 300 fold), but there was no significant reduction in SMC cells. The SMC cell type-specific expression regulation role of Exon 1 showed no more effects when the 5' upstream sequence extended to -748 and further region.

The MYOS core promoter sequence and its positive, negative or cell type-specific regulatory elements flanking sequences were used for the construction of a series of novel eukaryotic expression vectors, the basic unit of which offers the advantages of no limitation on gene size and copy numbers for stable and long term expression of cloned genes in a broad range of cell types of various species with compatible high expression levels but removes the possibility of promoter inactivation that occurs frequently with viral promoters in vivo.

The present invention provides the use of the rabbit smooth MHC gene '5 upstream sequence as promoter in the expression of reporter genes or other genes for transient expression studies or the establishment of genetic engineering cell banks in a wide range of mammalian host cells which can be used in basic research areas,

commercial applications as well as in human gene therapy.

Accordingly, this invention provides methods of gene expression for applications such as studies on the regulation of genes in eukaryotic cells and more specifically in vascular SMC cells, and the specific expression of genes with antiproliferative, antichemotactic or antithrombotic activities for human gene therapy in cardiovascular diseases and suitable for both ex vivo and in vivo gene delivery strategies.

This invention also provides a basis for the construction of therapeutic genes which can be easily transferred and expressed into a wide range of mammalian host cells or tissues in vitro and in vivo, for example for utilization in the treatment and preventation of other diseases such as cancer and a variety of inherited or acquired diseases. As is well known, SMC cells are one of the most promising cell type in long term delivery of therapeutic molecules or "gene drugs" to the whole body in future human gene therapy.

In another aspect, this invention provides novel eukaryotic expression vectors which are driven by a strong mammalian cellular promoter rather than retrovirus or other viruses and, therefore, have the advantages of being safer, more stable and giving more efficient

expression in mammalian cells, particularly for expression in vivo, bearing in mind the regulations and safety issues involved in human gene therapy.

In addition, the invention provides the use of this MYOS promoter as a probe, for screening, isolating and cloning purposes in human VSMC or VSMC of other species. The characterization of this new promoter and its regulatory elements and regulatory mechanisms will in turn enrich knowledge of the regulation of VSMC functions, like those of proliferation, migration and phenotype modulation. Also it will provide a basis for the development of second generation SMC specific expression vectors.

Furthermore, this invention provides the use of the MYOS promoter, optionally modified by subtraction of sequences or addition of cis-acting elements or combination with other vector types, e.g. polymerase III expression systems, retroviral vectors, adenoviral vectors etc, to regulate the transfection efficiency in different locations or cell-cycle phases, expression levels, periods, tissue-specificities etc, and which can be provided in any suitable form appropriate to the protocol of administration or the needs of a patient in gene therapy or as in vitro phenotype markers in clinical diagnosis systems.

A further example of the use of this invention as a diagnostic tool, is the construction of vectors containing marker genes for vascular SMC phenotype diagnosis purposes, and the in vitro testing for the response of patients to gene therapy targeted for example to inhibit human VSMC proliferation in atherosclerosis and intimal hyperplasia.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the restriction enzyme map of the MYOS promoter sequence.

Figure 2 illustrates the MYOS promoter sequence.

Figure 3 illustrates the putatitive regulatory-transcription factor binding sites in the MYOS promoter sequence.

Figure 4 illustrates the structures of the nested 5' deletion mutants used in the construction of pCAT-MYOS plasmids and the relative CAT activity levels in transfected rabbit VSMC cells.

Figure 5 illustrates the CAT activity levels obtained by autoradiography in primary rabbit VSMC

cells transfected with pCAT-MYOS constructs.

Figure 6 illustrates the structures of the pTRm1 series vectors - universal eukaryotic episomal expression vectors.

Figure 7 illustrates the structures of the pTRm2 series vectors - universal eukaryotic expression vectors.

Figure 8 illustrates the construction of pCAT-MYOS-Exon 1+/- plasmids.

Figures 9a, 9b and 9c illustrate the CAT activity levels obtained by different cell types transfected by pCAT-MYOS-Exon 1+/- constructs in (a) rabbit SMC (RSMC), (b) rat skeletal myoblasts (L6) and (c) human dermal fibroblasts (HDF).

Figure 10 illustrates the structure of the pTRm1S vector - SMC cell type-specific episomal expression vector.

Figure 11 illustrates the structure of the pTRm2S vector - SMC cell type specific expression vector.

Figure 12 illustrates the construction of pTRm2-β-

gal plasmid for in vivo expression studies.

Figures 13a, 13b and 13c illustrate β-gal activity expression in vivo - rabbit artery transduced by liposome pTRm2-β-gal plasmid DNA with double balloon catheter system.

DETAILED DESCRIPTION OF THE INVENTION AND PREFERRED EMBODIMENTS

In relation to Figures 1-13 the following materials and methods were used.

Materials: [14C] chloramphenicol (40-60 mCi/mmol) and [α-35S]-dATP were purchased from Amersham. Enzymes used in plasmid construction were obtained from Boehringer Mannheim Biochemicals and Promega. Plasmids pCATbasic, pCAT promoter, pCAT enhancer, pCAT control, pSV-β-gal, were from Promega. Plasmid pUC119 was from Amersham. Plasmids pREP9 and pRc/RSV were from Invitrogene. Bacterial chloramphenicol acetyltransferase (CAT enzyme) and β-galactosidase were from Promega. Acetyl coenzyme A and other chemicals were from Sigma.

Cell Cultures: The rabbit kidney epithelial cell line RK13, the skin fibroblast cell line RAB-9 and the rat skeletal myoblast cell line L6 were obtained from ATCC.

Rabbit smooth muscle cells (RSMC) were obtained from the thoracic aorta of 8-10 weeks old rabbits as described (27). Human umbilical vein endothelial cells (HUVEC), human aortic or vein smooth muscle cells (HSMC) and human dermal fibroblasts (HDF) were obtained by enzymatic digestion of tissues as described (28,29). RK13, RAB-9, L6, HSMC, RSMC and HDF cells were cultured in DMEM medium supplemented with 10 - 20% fetal calf serum (FCS), L-glutamine (2mM), fungizone (0.25 ug/ml), penicillin (100u/ml) and streptomycin (100 ug/ml) (all from Gibco) at 37°C under 10%CO_2. HUVEC were cultured in M199 medium with the same supplements plus endothelial cell growth factor (20 ug/ml) and heparin (80 ug/ml) with gelatin pre-coated flasks at 37°C under 7% CO_2.

Restriction Mapping: Standard protocols were followed for all techniques used. Plasmid pUC119-MYOS constructs for restriction mapping were obtained by cloning the PCR-derived fragment of the 5' upstream region of the rabbit smooth MHC gene (36) into pUC119 (Sal I site). The restriction map of this fragment was obtained by digestion with various restriction enzymes.

DNA sequence analysis: Sequence analysis of ds DNA of the recombinant plasmids was done by dideoxy chain termination method (30) using Sequenase (USB). The Wisconsin GCG program was used for the DNA sequence

assembly and analysis, i.e. a search of the homology sequences in GenEMBL banks showed the novelty of this sequence, and identified the putatitive vertebrate-encoded transcription factors binding sites (31).

The restriction map and the sequence of the MYOS promoter are shown in Figs. 1 and 2 respectively. As shown in Fig. 3, by searching with 154 transcription factor binding sites, we have found a redundancy of multiple binding sites in the MYOS promoter sequence. Such multiplicity of binding sites suggests the presence of complex mechanisms which may regulate the differential and tissue-specific expression of the smooth MHC gene.

Plasmid construction: The 2.0 kb PCR fragment was obtained by using sunthetic oligonucleotide 5'CTCCAAGTGGGGGGCGCGCGGAGAAACTGGACGGGGAGCGCGGGTCTGGCACA CCTCCCAGGCCGGGGCGTCCAAGGCT 3', which contains the entire first exon sequence of the smooth MHC gene (3' to 5' orientation), to do 5' extension PCR. This fragment, after purification, was cloned into pUC119 vector to construct the parental plasmid pUC119-MYOS used for deletion mutants constructions.

pUC119-MYOS plasmid was linearized by digestion with Xba I (partial digestion) and Kpn I restriction enzymes. Unidirectional deletion mutants were obtained by

exonuclease III digestion method using the Erase-a-base system (Promega). After restriction enzyme mapping and DNA sequencing check, pUC119-MYOS 1 - 8 plasmids which contain the 5' upstream region of smooth MHC gene from -1, -110, -180, -390, -426, -504, -638, -748, -950 to +78 respectively, were chosen for the further constructions of pCAT-MYOS plasmids.

pCAT-MYOS-EXON 1+/- plasmids were constructed by purifying MYOS insert fragments from pUC119-MYOS 1 - 8 plasmids, subcloning into the pCATbasic vector (Promega) at the Sal I site by blunt-end ligation, to obtain the pCAT-MYOS-EXON 1+ mutants with the insert fragments being subcloned at both orientations. The pCAT-MYOS-EXON 1- mutants were obtained by BssH II and Xba I restriction enzymes digestion and religation. All mutants constructions were confirmed by DNA sequencing.

The plasmids pREP9 and pRc/RSV (Invitrogene) were used as the backbone for the construction of pTRm series vectors. The RSV-LTR sequence was removed by restriction enzymes digestion (Nru I, Hind III), and replaced by blunt-end ligation of MYOS promoter fragments with various regulatory element sequences.

The pTRm2-β-gal plasmid was constructed by inserting the bacterial lacZ gene fragment into the multi cloning site

of pRc-RSV vector (Hind III, Xba I), then removing the RSV-LTR and replacing it with MYOS promoter fragments by blunt-end ligation.

Cell transfection: All cell transfection experiments were carried out using the electroporation method (9,33) - BIO-RAD Gene Pulser System. Monolayer growing cells were plated at 70-80% confluency in culture medium one day before the electroporation. Trypsinized cells (0.05% trypsin, 0.02% EDTA) were washed once with Electroporation buffer (137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6mM D-glucose, 21 mM HEPES, pH7.1) and resuspended in the same buffer at 2 x 10^6 - 10^7 cells/ml. Aliquots of 0.5 ml cell suspension were transferred into 0.4 cm electroporation cuvettes. Plasmid DNA (20 - 40 ug) was added and the cells were electroporated at 260V/960 uF (SMC, fibroblasts, myoblasts) or at 230V/960 uF (endothelial cells). After electroporation, the cells were replated in 10 cm^2 dishes with 10ml growth medium and incubated for 48 hr at 37°C/10% CO_2. Cells were subsequently harvested for assaying the enzyme activity and protein content. The transfection efficiency was normalized by co-transfection with a second reporter gene vector i.e. pSV-β-gal or pGLcontrol (luciferase) (Promega).

CAT enzyme activity: CAT expression was assayed as

described (8) with some modifications. Briefly, cell extracts in 0.25 M tris-HCl (pH 8.0) obtained by freeze-thaw were heated at 60°C for 10 min. for the inactivation of endogenous acetylase. Aliquots of 115 ul were incubated with 5 ul of 14C-chloramphenicol (Amersham, 0.025 mCi/ml) and 5 ul of n-butyryl coenzyme A (5 ug/ml, Sigma) at 37°C for 60 min. The reaction products were extracted with 300 ul xylene (Aldrich) and subsequently used for Thin Layer Chromatography (TLC) or Liquid Scintillation Counting (LSC).

TLC Assay: 5 ul of xylene cell extract was taken, vacuum dried, redissolved in 10 ul MeOH and chromatographed on silica gel 60F-245 TLC plates (Merck) using $CHCl_3$:MeOH (97:3). CAT enzyme products were detected by autoradiography (7-10 days exposure) using X-ray film (Fuji).

LSC Assay: 250 ul of xylene cell extract was taken, back extracted twice with 0.25 Tris-HCl pH 8.0, 100 ul. After the extraction, 200 ul of the upper organic phase solution which contained the butyrylated chloramphenicol products, were transferred into 3 ml scintillant (Optiphase "High Safe" II, LKB) and counted in a Beckman LS 6000 SE Counter.

β-gal enzyme assay: Samples of cell extracts (without

60°C heating) which were co-transfected with pSV-β-gal plasmid, were assayed for the β-gal activity as previously described (34).

Protein content assay: The protein concentration of cell extracts were determined using a protein assay kit (BIO-RAD) based on the methods of Bradford (35).

Luciferase activity assay: Samples of cell extracts (without 60°C heating) which were co-transfected with pGLcontrol (luciferase) plasmid, were assayed for the luciferase activity using the luciferase assay system from Promega.

In vivo Direct gene delivery system in rabbit model: The femoral artery of adult rabbits (3.0 kg in weight) was exposed under anaesthesia, any superficial branches were ligated so as to provide a 2.0cm isolated segment, a double balloon catheter (USCI) was introduced into the vessel via a small distal arteriotomy. Inflation of the proximal and distal balloons created a central space that allows for the infusion of liposome/DNA solution through an instillation port. 60ug pTRm2-β-gal or pSV-β-gal plasmid DNA were mixed with liposome (DMRIE/DOPE, VICAL, liposome: DNA = 1:40 [molar ratio]) diluted by Opti-MEM medium (GIBCO, serum-free) to a total volume of 0.4 ml. The solution was instilled into the central space for 30

min, followed by removal of the catheter and restoration of blood flow. Three to seven days later, the arterial segments were removed and examined after histochemical staining, the blue coloration indicating β-galactosidase expression. This double balloon catheter system and the histochemical assay method of β-gal expression in the vessel wall were based on the method of Nabel (38,39).

According to the present invention, we have identified and cloned the 5' upstream promoter region of the rabbit smooth MHC gene (Figs. 1 and 2). This fragment has strong promoter activity (named MYOS promoter) which can drive CAT (and other) gene expression in mammalian cells to levels up to for example 5- to 10-fold higher than those obtained with SV 40 promoter (Fig. 4 and 5).

Genbank and EMBL bank searches showed that this MYOS promoter sequence is new, as shown in Fig. 2. This fragment encompasses at its 3' end, the entire first exon with a sequence identical to that previously reported (36). 5' upstream of the transcription initiation site it contains a consensus TATA box sequence at position -30. No CAAT box was found. The 5' flanking region contains G+C rich (76%) sequences and is enriched with a relatively large number of putatitive transcription factors binding sites as well as repeats sequences (Figs. 2 and 3), suggesting that they may be involved in the

transcriptional regulation mechanisms which may determine the developmental and tissue-specific expression of the smooth MHC gene.

Localization of core promoter and regulatory elements regions were reached by transient expression studies with pCAT-MYOS constructs transfected into rabbit and human vascular SMC cells and non-smooth muscle cells of different species. Co-transfection with pSV-β-gal plasmid was used for the normalization of transfection efficiency. The MYOS core promoter was identified upstream to position -180, which produced as high as -92% CAT activity of the pCAT control vector in which CAT gene expression was driven by the SV40 promoter/enhancer. The highest expression level of CAT gene driven by MYOS promoter mutants occured with pCAT-MYOS 4+ and pCAT-MYOS 7+, which were reached > 100 - 103% CAT activity of the pCAT control vector, suggesting that the MYOS promoter is a strong promoter and the existance of positive elements in its proximal upstream region (-390 to -180) and distal upstream region (-748 to -638). All pCAT-MYOSop (reverse orientation) constructs showed very low levels of CAT expression, suggesting these sequences contain promoter activity but not enhancers.

pCAT-MYOS-EXON 1- constructs from which the first exon sequence (+1 to +78) is deleted showed that in rabbit SMC

Exon 1 has a positive cis-regulatory function for the full activity of the MYOS promoter up to position -504. Without the Exon 1 sequence, the MYOS promoter activity up to position -638 was greatly reduced in other cell types (i.e. L6 and HDF cells, more than 300 fold), but no significant reduction was found in SMC cells. The SMC cell type-specific expression regulation role of Exon 1 showed no more effects when the 5' upstream sequence extended to -748 and up to -950.

An in vivo gene deliver system in the rabbit artery model showed that these MYOS promote-based new eukaryotic expression vectors can be easily transduced into vessel wall and drive reporter gene (such as β-gal) expression in vivo. The primary investigation showed that the expression level from pMYOS-β-gal was higher than pSV-β-gal (Fig. 13).

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CLAIMS:

1. The nucleotide sequence from -950 to +78 bp of the rabbit smooth muscle myosin heavy chain gene.

2. The nucleotide sequence of claim 1 for use as a promoter in the expression of one or more genes, especially reporter genes in a mammalian host cell.

3. The nucleotide sequence of claim 1 for use in the construction of a eukaryotic expression vector comprising a reporter or marker gene.

4. A eukaryotic expression vector comprising the nucleotide sequence of claim 1 or a portion thereof.

5. An expression vector according to claim 4, selected from any of those vectors disclosed herein with reference to the accompanying drawings.

6. A plasmid comprising the nucleotide sequence of claim 1, or a portion thereof.

7. A plasmid according to claim 6, selected from any of those plasmids disclosed herein with reference to the accompanying drawings.

8. A mammalian host cell transfected with a gene comprising the sequence of claim 1 as the promoter therefor.

9. A cell according to claim 8, which is selected from rabbit or human or other vascular smooth muscle cells, human dermal fibroblasts, rabbit skin fibroblasts, rabbit kidney epithelial cells or rat skeletal muscle myoblasts.

10. A method of initiating and/or regulating expression of a gene in a mammalian host cell by use of a promoter therefor which comprises the nucleotide sequence of claim 1.

11. A method according to claim 10, wherein the host cell is a human cell.

12. A method according to claim 10, wherein the said gene is that which codes for chloramphenicol acetyl transferase (CAT) or β-galactosidase or the firefly luciferase gene.

13. A method according to claim 10, wherein the host cell is a rabbit or human or other vascular smooth muscle cell, human dermal fibroblast, rabbit skin fibroblast, rabbit kidney epithelial cell or rat skeletal muscle myoblast.

14. Use of the nucleotide sequence of claim 1 as a promoter in the expression of one or more genes, especially one or more reporter genes, in a mammalian host cell.

15. Use of the nucleotide sequence of claim 1 in the construction of a eukaryotic expression vector for the transient or stable expression of a foreign gene in a mammalian host cell.

16. Use of the nucleotide sequence of claim 1 in the construction of a vector comprising one or more marker genes for vascular SMC phenotype diagnosis.

17. Use of the nucleotide sequence of claim 1 as a gene probe.

18. Use of the nucleotide sequence of claim 1 in human gene therapy.

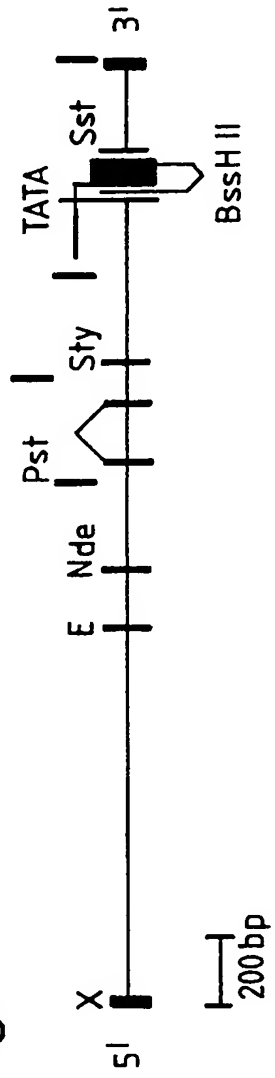


Fig.1.

Fig.2.

-950 GAGGGTCTTC CGAGGACCCT TCCAAACCCG ACGTGGGAAG CCTGGCGAAA

-900 GGATGATTTT GGTGCAAAAA ACCCCTGACA TCCATGCCTC CGAGGGGTCT

-850 TGCAAAAAGT TCATATGGGA AACGCATGTT ATGAAAAAGT GTGCATGGAT

-800 CTCAATGTTC TCTTTCCCCC ATCAAAATAA AGTCAGCTTT TAATTCCTTC

-750 TGGCCGTGAA CTTTCTGAAC GACCCTGGGA CGCAGAGACC GAGGCACGAG

-700 CGGGAGAGCG AGGGGCCGCG ATGGTGACCG AGGCAGTGAG AACCCCCCGG

-650 GCGCCCACGG CCAGGGAGTC GCGCGGGGTC GAGGCCGCGT CCCCCAGTCC

-600 CGCAGCGCCC CCTCGGCAGC GGGACCCGCC GGCTGGGCTG GGGTGCGGAC

-550 GACCCGCCTG GCACGCGACC GGTTATCCCC GCCCCCTCCT CTGTTTAGCA

-500 GTGCGACCCC GGGCTCGGGA AGGAGAGTGA CTCGGCCAAG GTCTCGCGTG

-450 GGGCCGAGGT CGCGGCGGCC GGACCGAAGT CGGGAAGGGC AGGCGCTGCC

-400 CGGTGCGCAC CCTGCCCGGC CTCGCACTTG GCCAAGTTCA GAAAATTCCC

-350 TTTCCCGACT CTTCCCTGCA GGCCCCGGGA GGGAGGTGCG CCAGCGTCCC

-300 CCCAGCGGGG GGAGCCGGAG GTTGCAGCCT GGGGTGCGAC TCCGGGGCGA

-250 ACTTCTCCAG CACCCCGTTC TCTAGGGACT CGGAGCCCCA GAGACCGCCG

-200 CTGTTCCGGG GTCCGGGAGC ACCCAGGGGA CAGACAGCGC TCTGGGCCGG

-150 GGCCAAAGCC GCACGCCTCG GGCCCTGCAG GGGGCGCTGC GGAGGGAGCG

-100 GAGCGAGACC CTCCCCCACC GCACCCCGTG GGCCGCCTCC CCCGTCCCAC

-50 CCGCGGGCAG CTCCGGGTGT ATAAAGAGCA GCGTCCGAGG CGCGCAGGGA

+1 GCCTTGGACG CCCCGGCCTG GGAGGTGTGC CAGACCCGCG CTCCCCGTCC

+51 AGTTTCTCCG CGCGCCCCCC ACTTGGAG

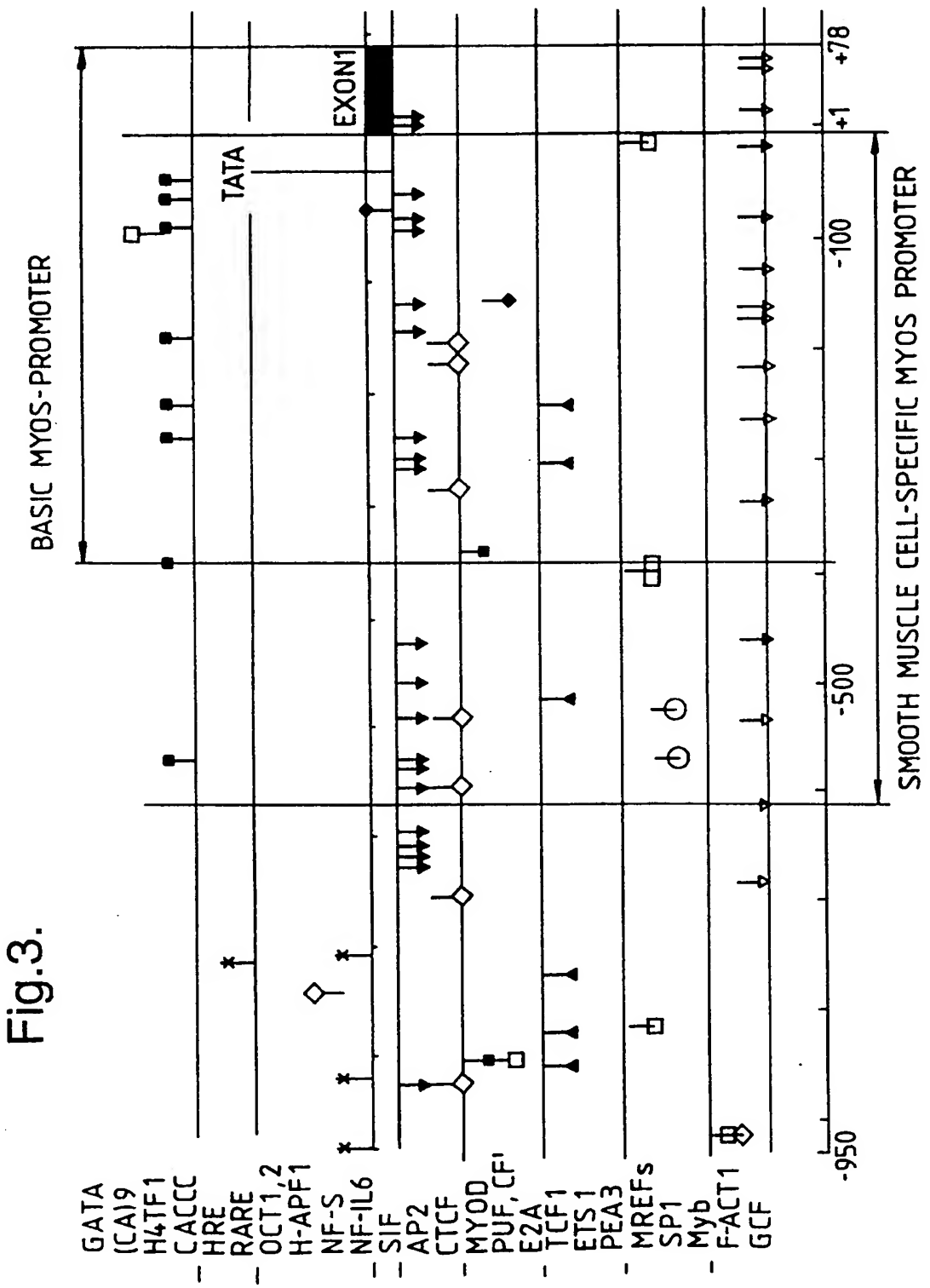

BASIC MYOS-PROMOTER
SMOOTH MUSCLE CELL-SPECIFIC MYOS PROMOTER
EXON1
TATA
+78
+1
-100
-500
-950
GATA
(CA)9
H4TF1
CACCC
HRE
RARE
OCT1,2
H-APF1
NF-S
NF-IL6
SIF
AP2
CTCF
MYOD
PUF, CF1
E2A
TCF1
ETS1
PEA3
MREFs
SP1
Myb
F-ACT1
GCF

Fig.3.

Fig.4.

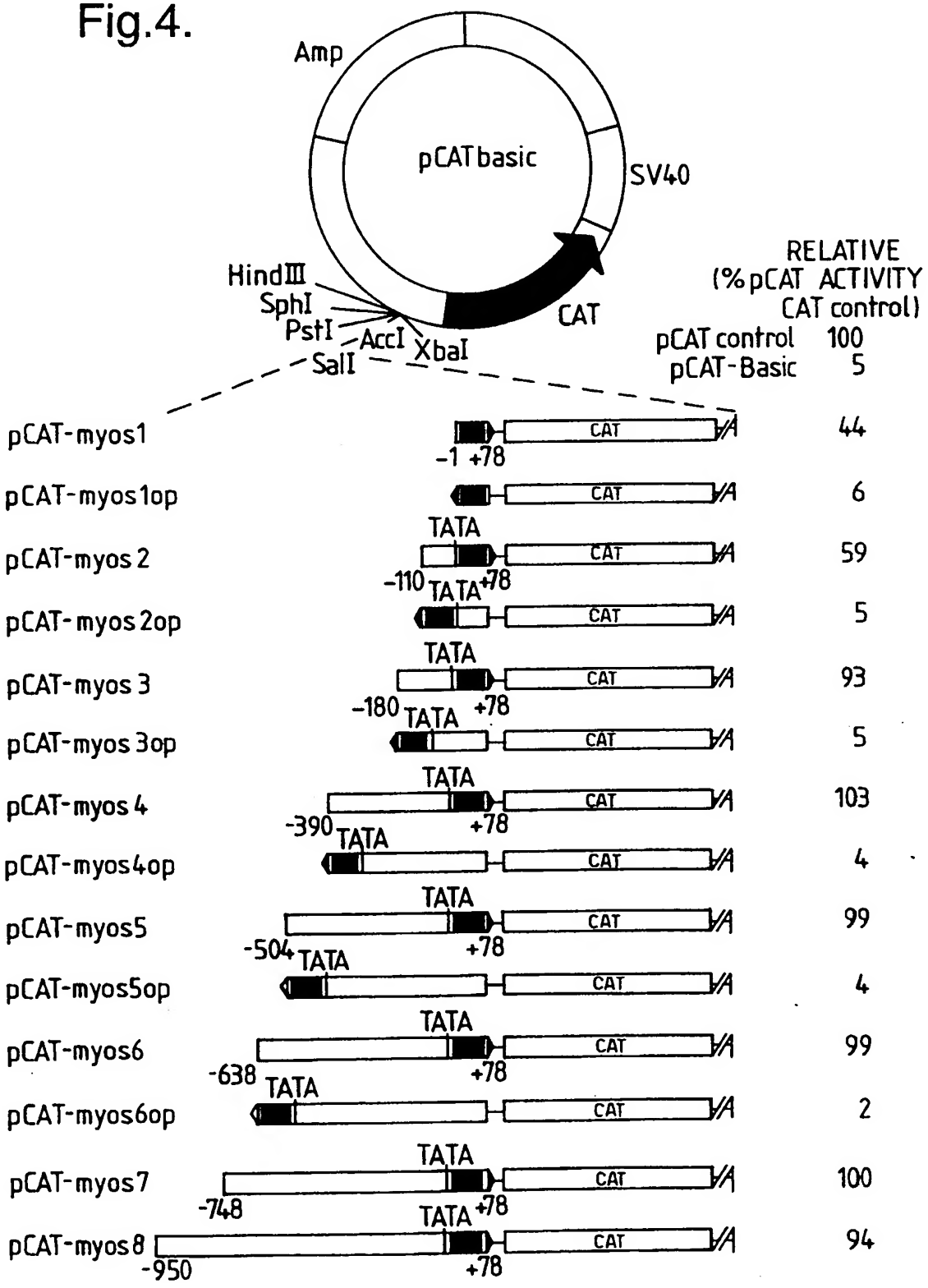

Amp
pCATbasic
SV40
CAT
HindIII
SphI
PstI
AccI
XbaI
SalI
RELATIVE
(% pCAT ACTIVITY
CAT control)
pCAT control 100
pCAT-Basic 5
pCAT-myos1
-1 +78
44
pCAT-myos1op
6
pCAT-myos 2
TATA
-110
+78
59
pCAT-myos 2op
5
pCAT-myos 3
-180
93
pCAT-myos 3op
5
pCAT-myos 4
-390
103
pCAT-myos4op
4
pCAT-myos5
-504
99
pCAT-myos5op
4
pCAT-myos6
-638
99
pCAT-myos6op
2
pCAT-myos7
-748
100
pCAT-myos8
-950
94

Fig.5.

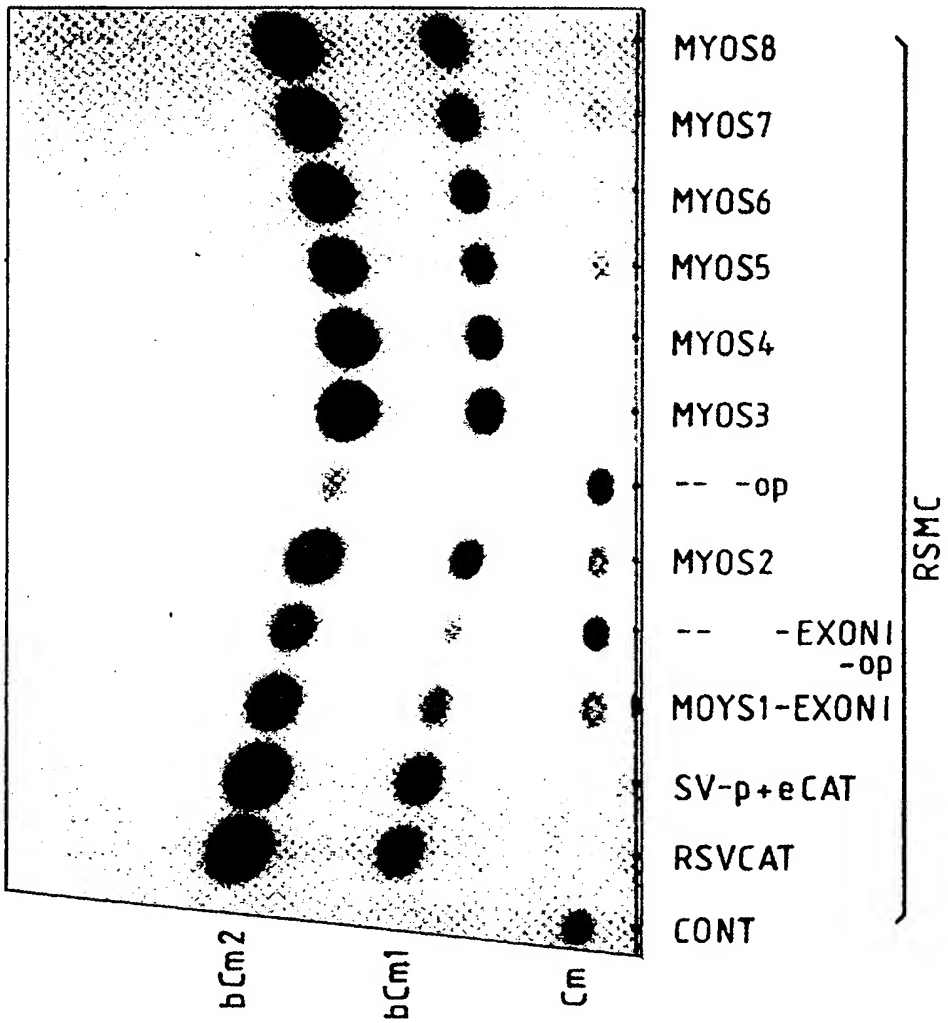

MYOS8
MYOS7
MYOS6
MYOS5
MYOS4
MYOS3
-- -op
MYOS2
-- -EXON1
-op
MOYS1-EXON1
SV-p+eCAT
RSVCAT
CONT
RSMC
bCm2
bCm1
Cm

Fig.6.

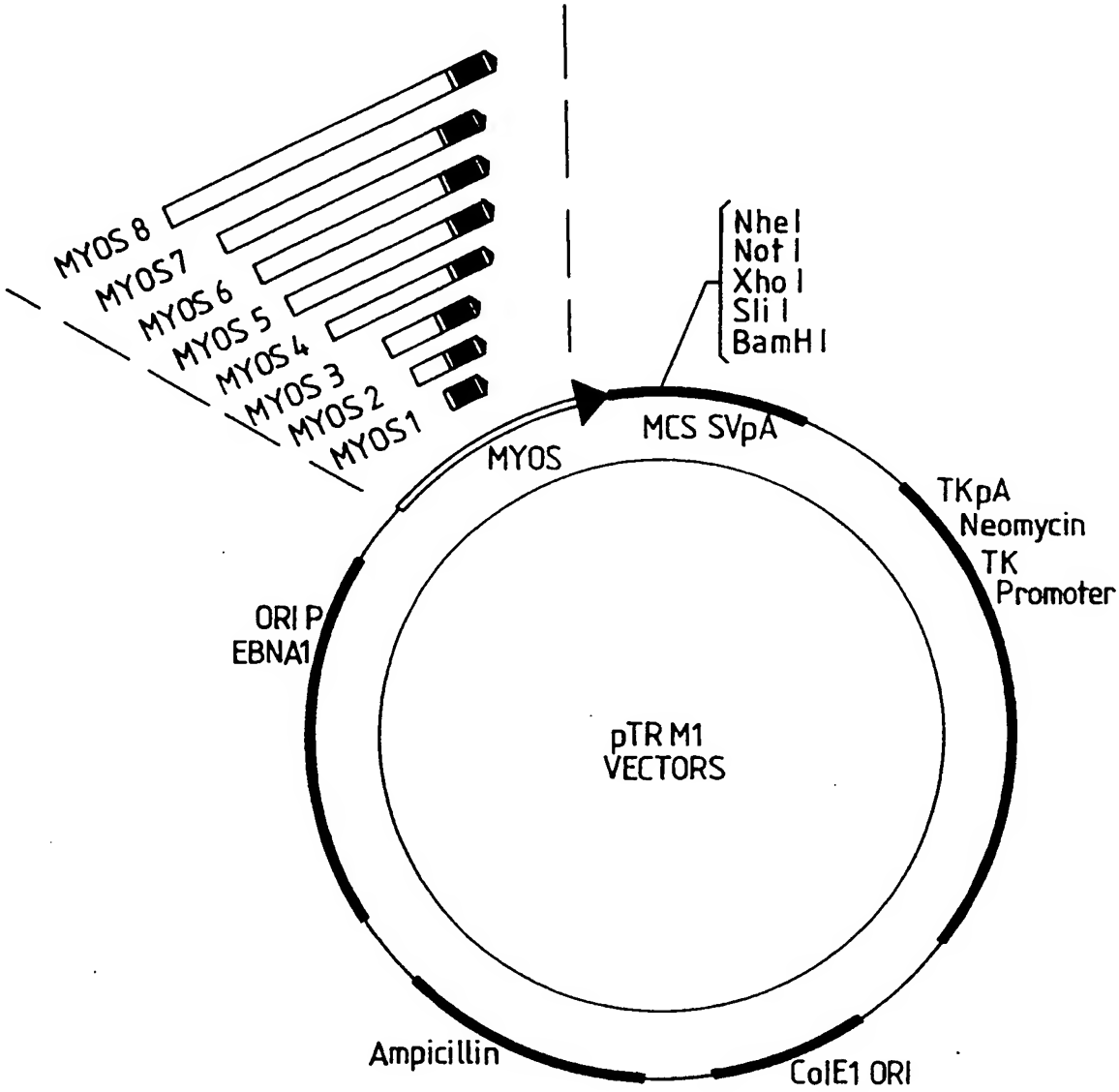

MYOS 8
MYOS 7
MYOS 6
MYOS 5
MYOS 4
MYOS 3
MYOS 2
MYOS 1
Nhe I
Not I
Xho I
Sli I
BamH I
MCS SVpA
MYOS
TKpA
Neomycin
TK
Promoter
ORI P
EBNA1
pTR M1
VECTORS
Ampicillin
ColE1 ORI

Fig.7.

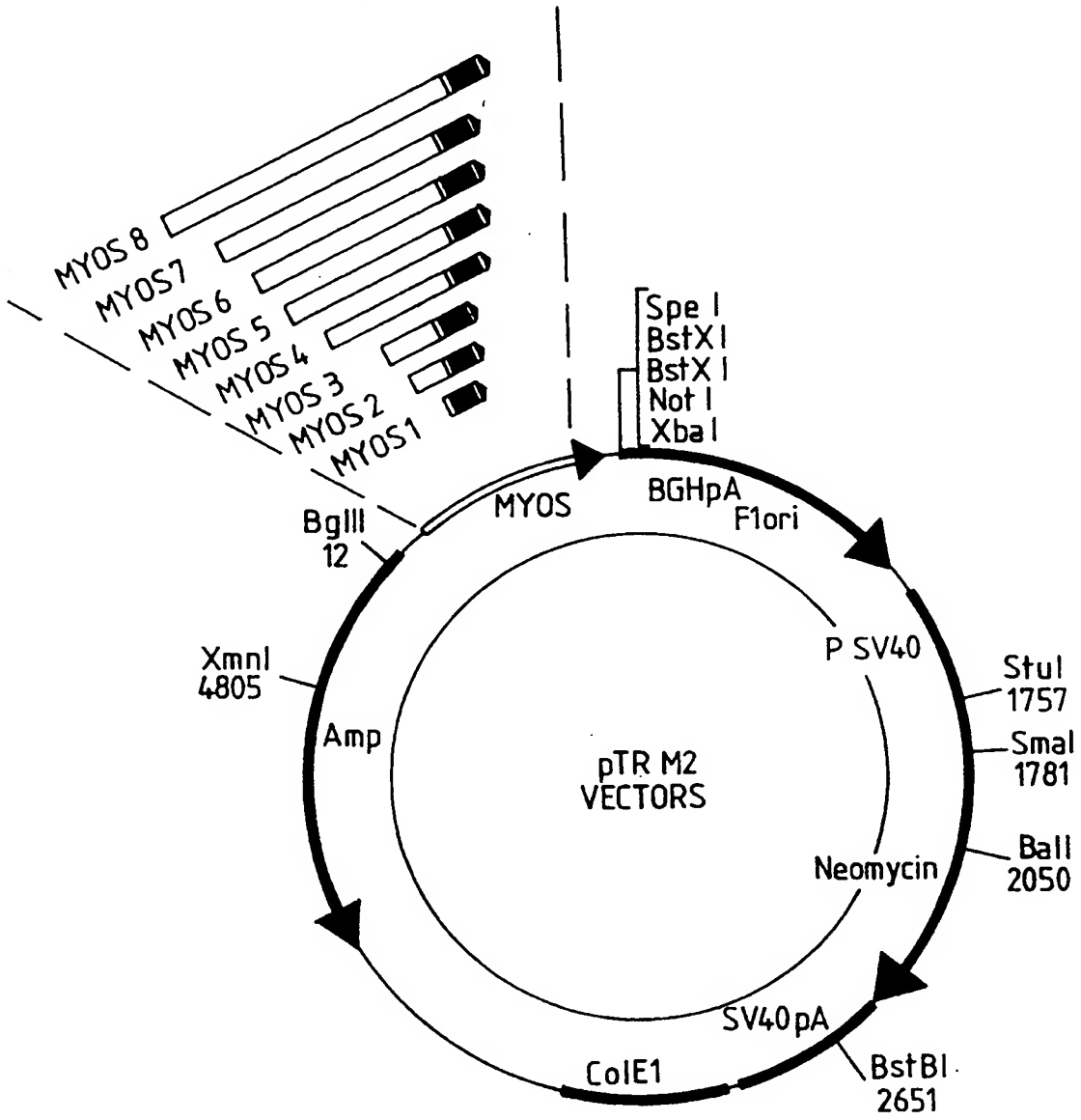
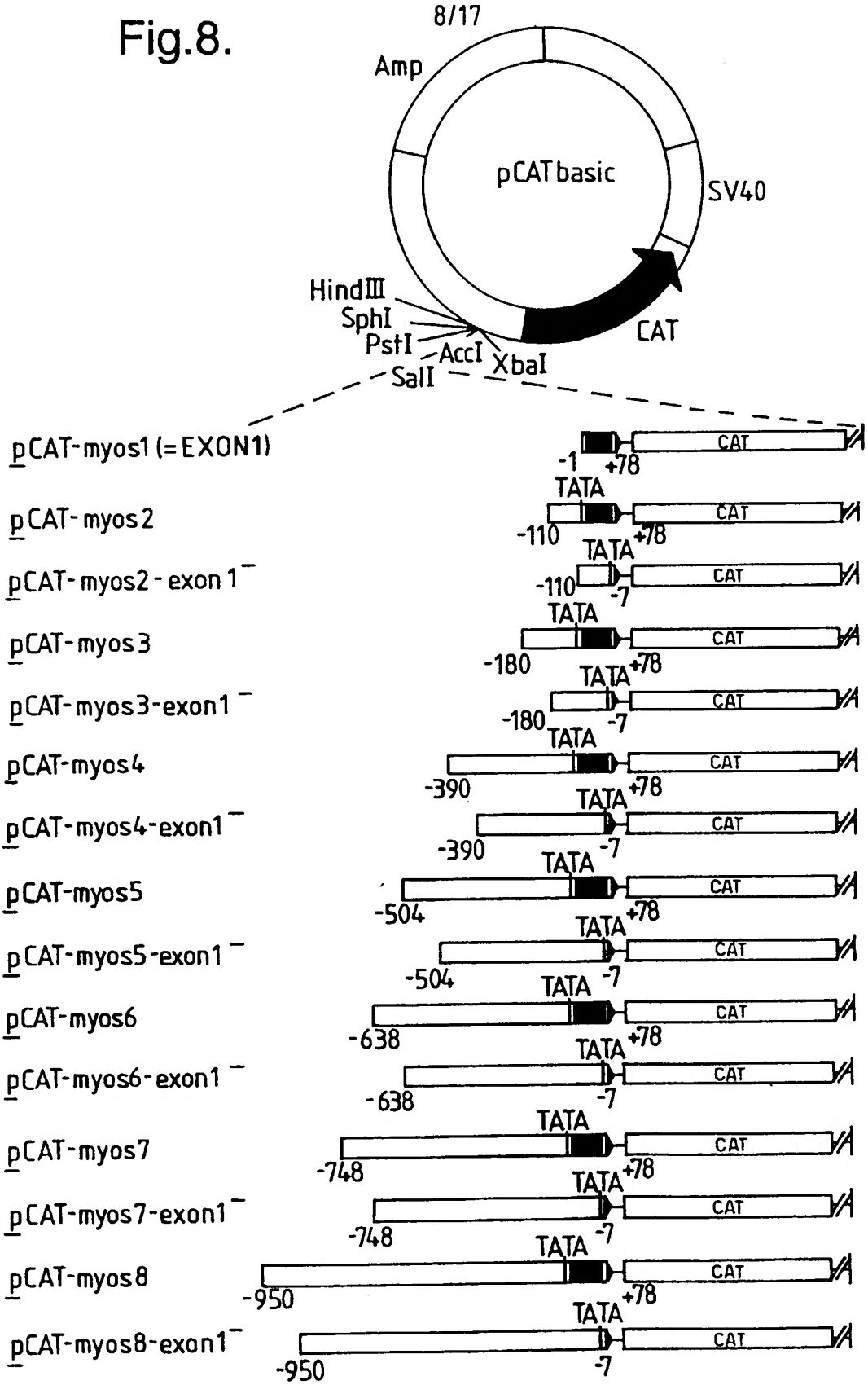

MYOS 8
MYOS 7
MYOS 6
MYOS 5
MYOS 4
MYOS 3
MYOS 2
MYOS 1
Spe I
BstX I
BstX I
Not I
Xba I
BGHpA
F1ori
MYOS
BglII
12
P SV40
StuI
1757
SmaI
1781
BalI
2050
Neomycin
SV40 pA
BstBI
2651
ColE1
Amp
XmnI
4805
pTR M2
VECTORS

Fig.8.


8/17
Amp
pCAT basic
SV40
HindIII
SphI
PstI
AccI
XbaI
SalI
CAT
pCAT-myos1 (=EXON1)
-1
+78
pCAT-myos2
TATA
-110
pCAT-myos2-exon1⁻
-7
pCAT-myos3
-180
pCAT-myos3-exon1⁻
pCAT-myos4
-390
pCAT-myos4-exon1⁻
pCAT-myos5
-504
pCAT-myos5-exon1⁻
pCAT-myos6
-638
pCAT-myos6-exon1⁻
pCAT-myos7
-748
pCAT-myos7-exon1⁻
pCAT-myos8
-950
pCAT-myos8-exon1⁻

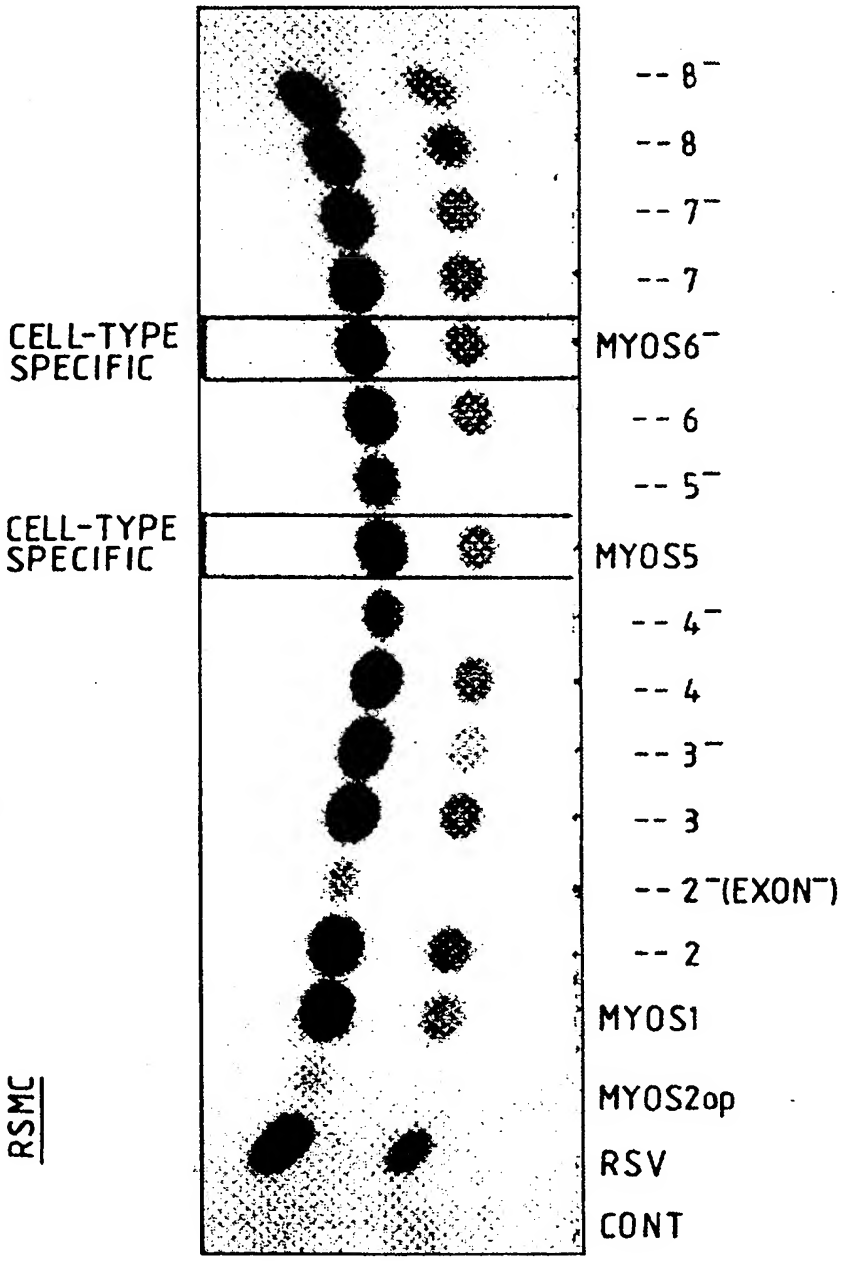

-- 8⁻
-- 8
-- 7⁻
-- 7
CELL-TYPE SPECIFIC
MYOS6⁻
-- 6
-- 5⁻
CELL-TYPE SPECIFIC
MYOS5
-- 4⁻
-- 4
-- 3⁻
-- 3
-- 2⁻(EXON⁻)
-- 2
MYOS1
MYOS2op
RSV
CONT
RSMC

Fig. 9a.

Fig.9b.

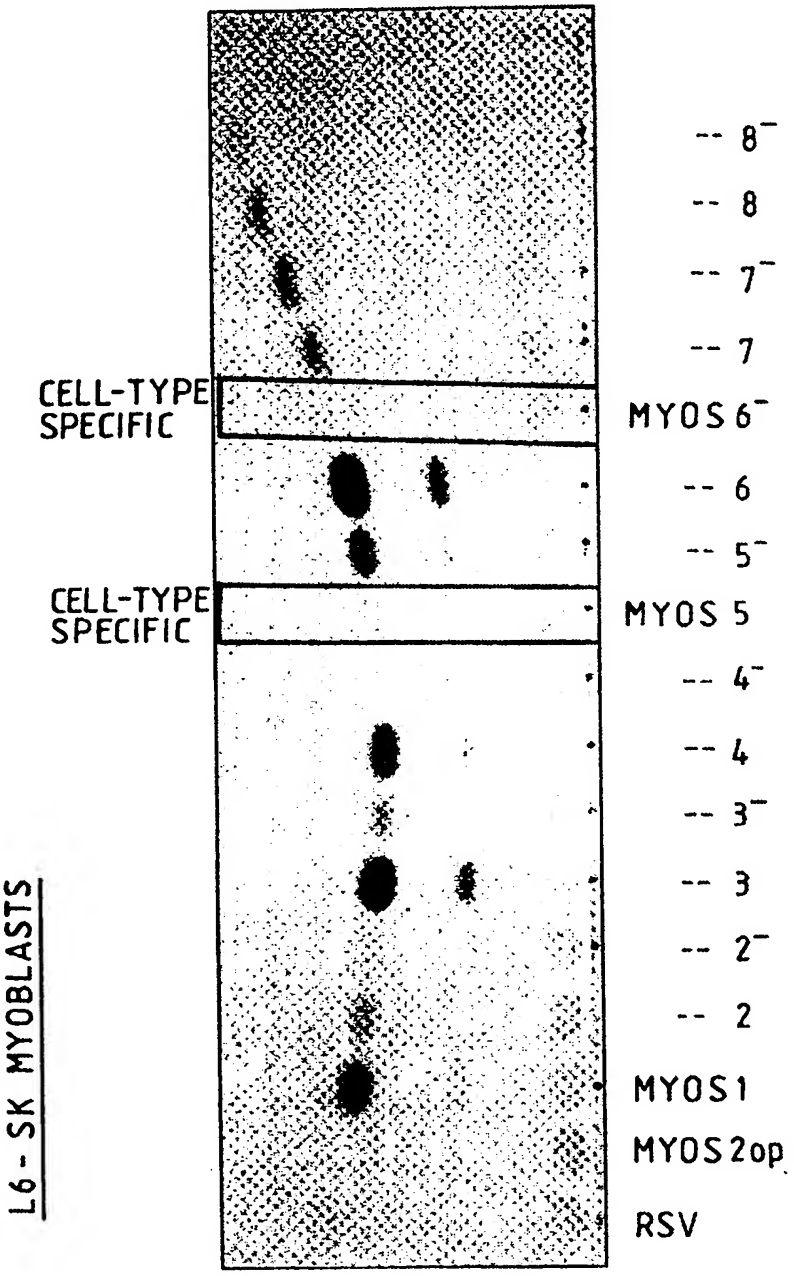

L6 - SK MYOBLASTS
CELL-TYPE SPECIFIC
CELL-TYPE SPECIFIC
-- 8⁻
-- 8
-- 7⁻
-- 7
MYOS 6⁻
-- 6
-- 5⁻
MYOS 5
-- 4⁻
-- 4
-- 3⁻
-- 3
-- 2⁻
-- 2
MYOS 1
MYOS 2op
RSV

Fig.9c.

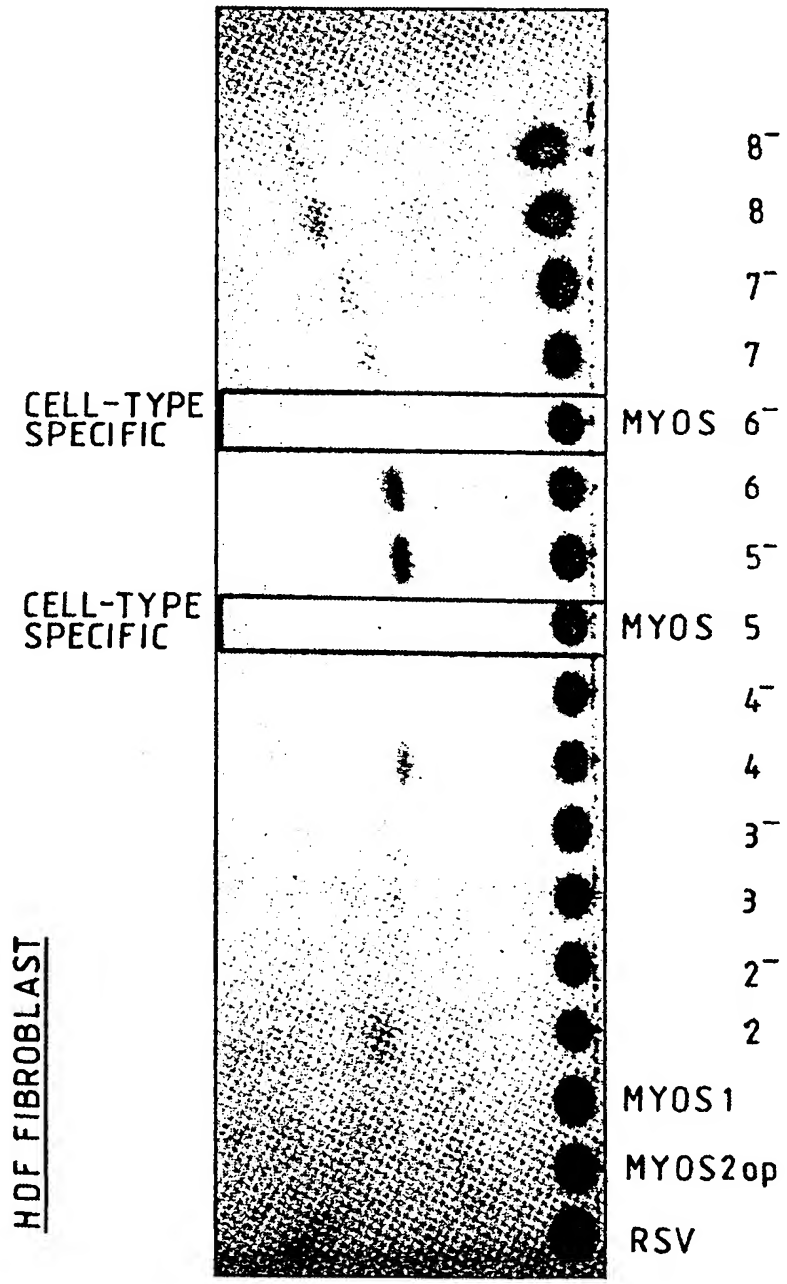
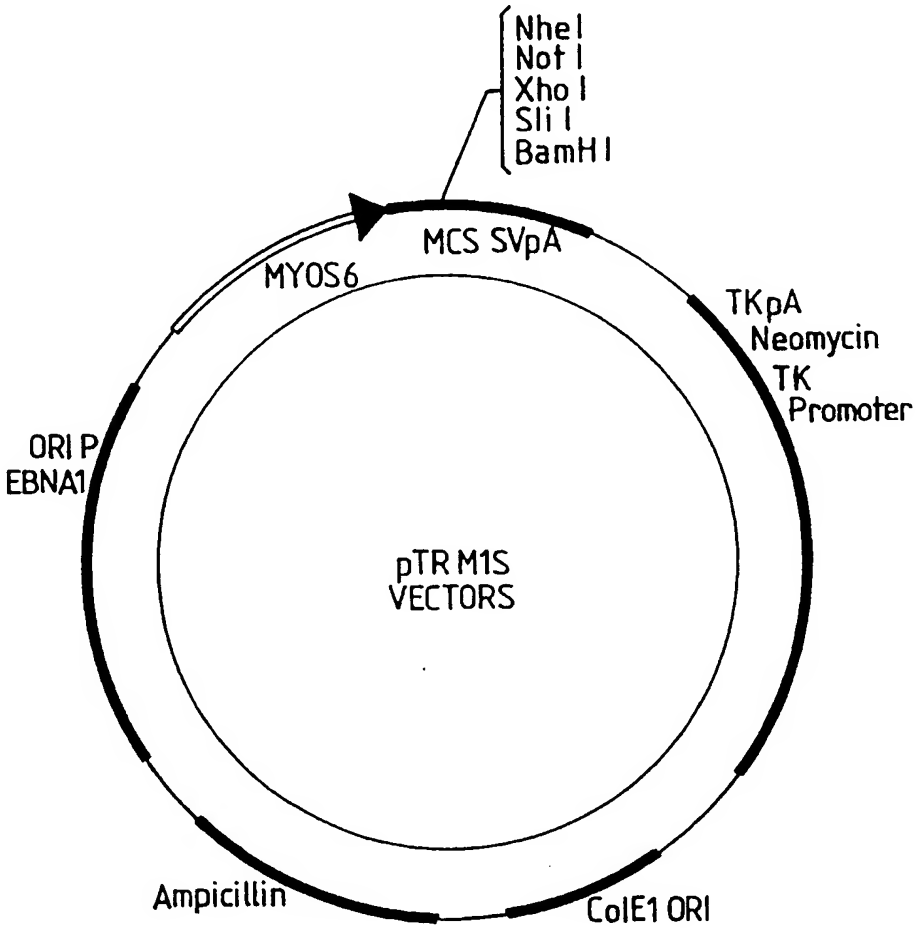

8⁻
8
7⁻
7
CELL-TYPE SPECIFIC
MYOS 6⁻
6
5⁻
CELL-TYPE SPECIFIC
MYOS 5
4⁻
4
3⁻
3
2⁻
2
MYOS1
MYOS2op
RSV
HDF FIBROBLAST

Fig.10.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Not I
Xho I
Sli I
BamH I
MCS SVpA
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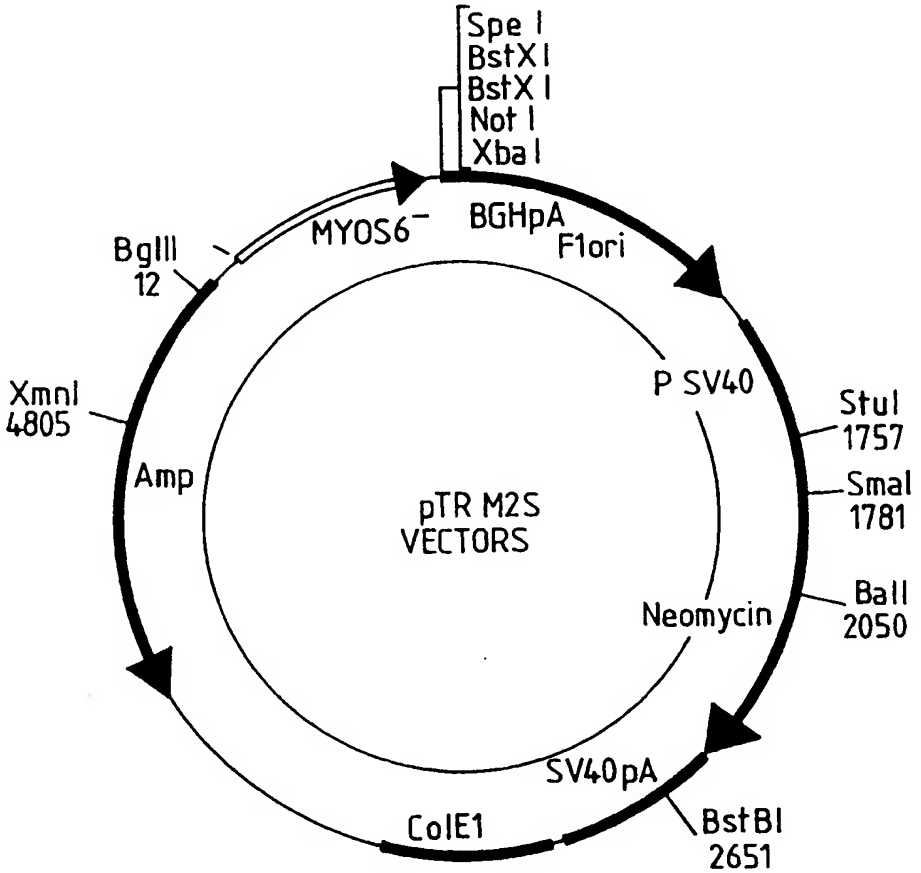
TK
Promoter
ORI P
EBNA1
pTR M1S
VECTORS
Ampicillin
ColE1 ORI

Fig.11.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BstX I
Not I
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BGHpA
F1ori
MYOS6
BglII
12
P SV40
StuI
1757
SmaI
1781
BalI
2050
Neomycin
SV40pA
BstBI
2651
ColE1
Amp
XmnI
4805
pTR M2S
VECTORS

Fig.12.

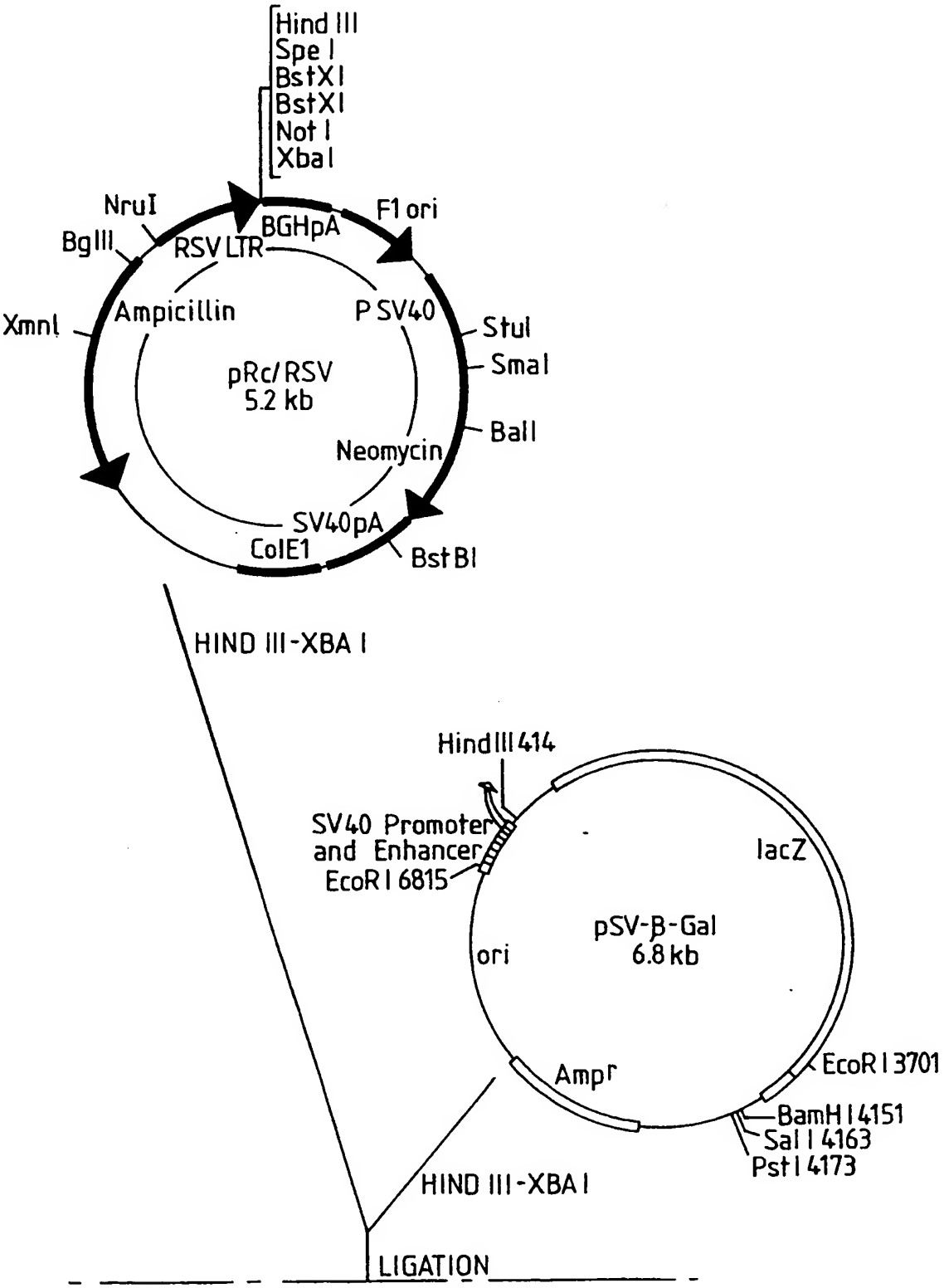
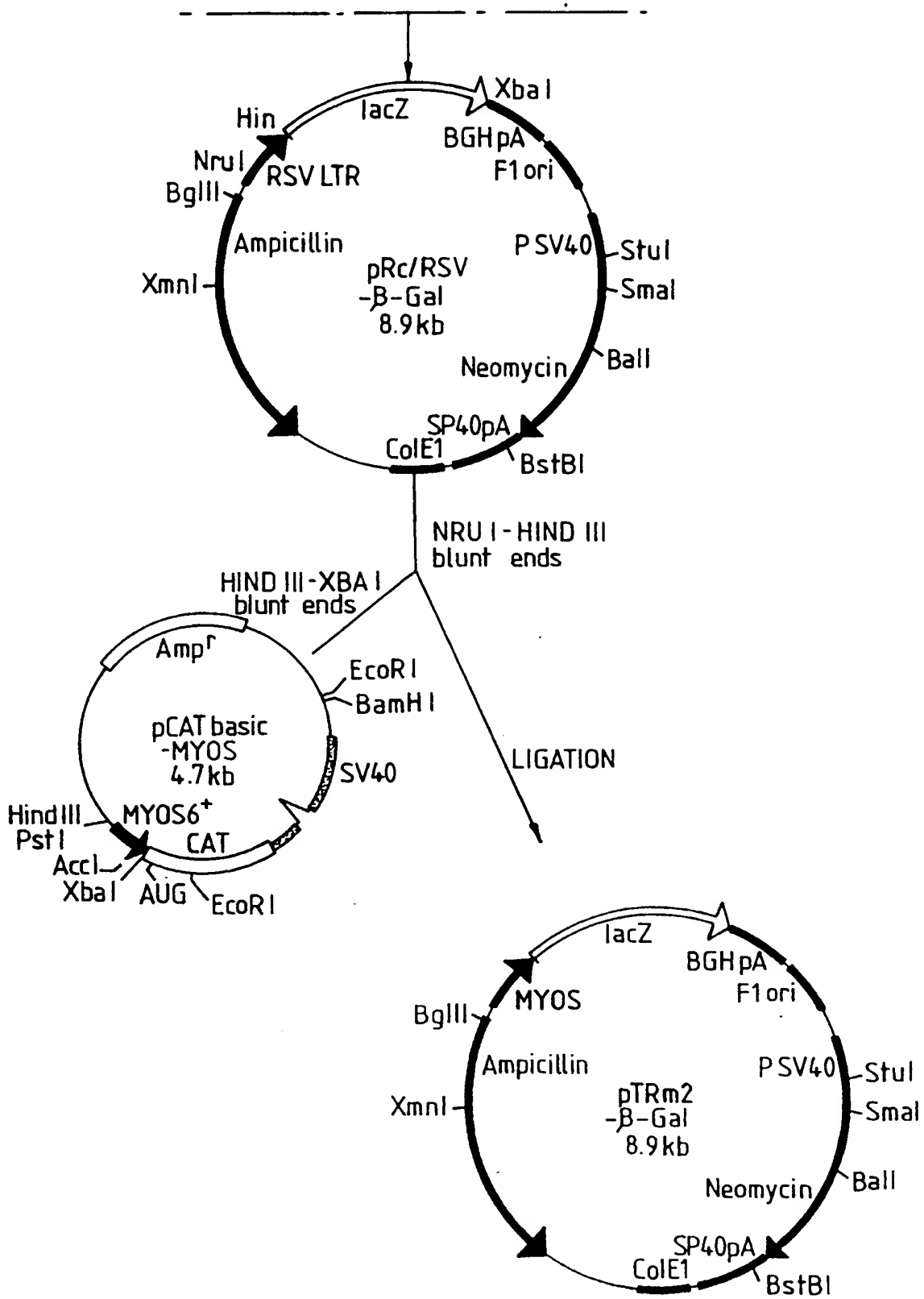

Hind III
Spe I
BstXI
BstXI
Not I
Xba I
NruI
BglII
RSV LTR
BGHpA
F1 ori
Xmn I
Ampicillin
P SV40
Stu I
Sma I
pRc/RSV
5.2 kb
Bal I
Neomycin
SV40pA
ColE1
BstBI
HIND III-XBA I
Hind III 414
SV40 Promoter
and Enhancer
EcoR I 6815
lacZ
pSV-β-Gal
6.8 kb
ori
Ampr
EcoR I 3701
BamH I 4151
Sal I 4163
Pst I 4173
HIND III-XBA I
LIGATION

Fig.12(Cont).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lacZ
Hin
BGH pA
F1 ori
Nru I
RSV LTR
Bgl II
Ampicillin
P SV40
Stu I
Xmn I
pRc/RSV
-β-Gal
8.9 kb
Sma I
Neomycin
Ball
SP40pA
ColE1
BstBI
NRU I - HIND III
blunt ends
HIND III - XBA I
blunt ends
Amp^r
EcoR I
BamH I
pCAT basic
-MYOS
4.7 kb
SV40
LIGATION
Hind III
MYOS6+
Pst I
CAT
Acc I
Xba I
AUG
EcoR I
lacZ
BGH pA
F1 ori
Bgl II
MYOS
Ampicillin
P SV40
Stu I
Xmn I
pTRm2
-β-Gal
8.9 kb
Sma I
Neomycin
Ball
SP40pA
ColE1
BstBI

Fig.13a.

RABBIT ARTERY NORMAL CONTROL

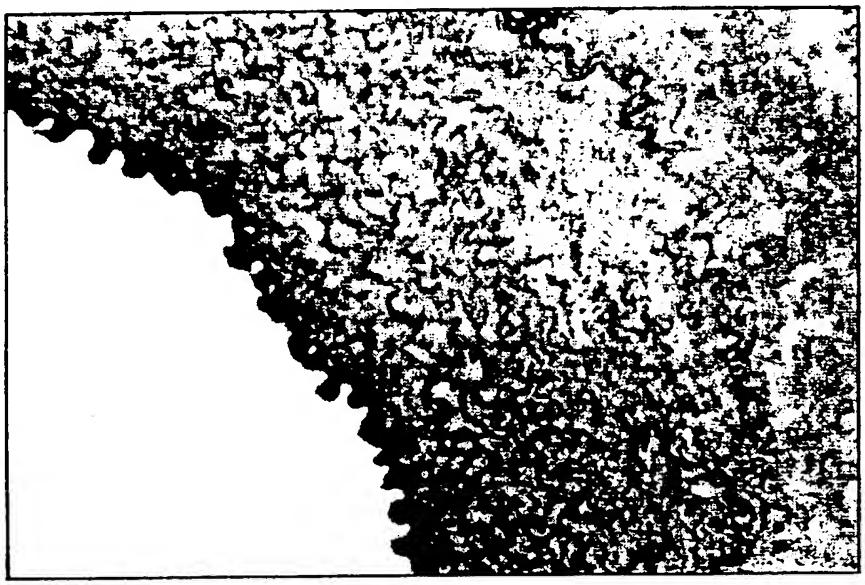
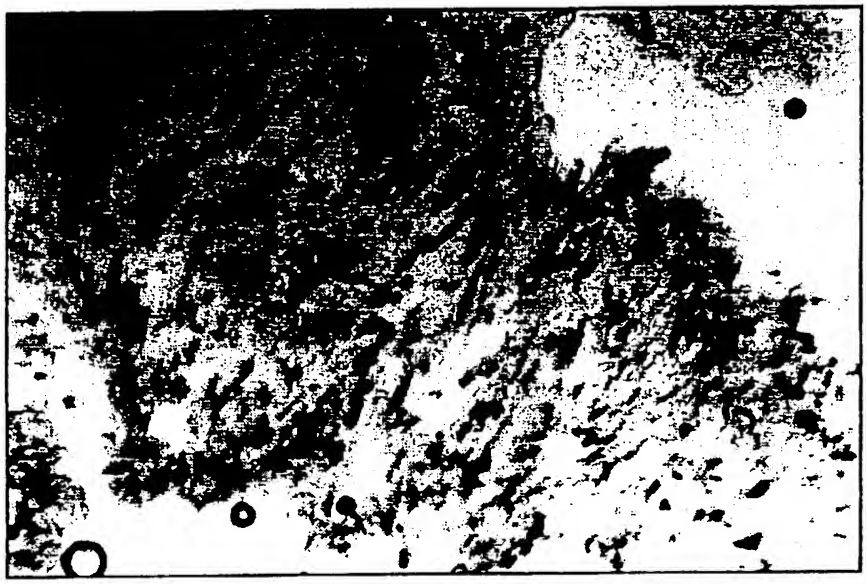


Fig.13b.

RABBIT ARTERY TRANSDUCED WITH pTRm2-B-gal PLASMID DNA (3 DAYS)

Fig.13c.

RABBIT ARTERY TRANSDUCED WITH pSV-B-gal PLASMID DNA (3 DAYS)



INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 94/00489

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N15/85 C12N15/11 C12N5/10 C12Q1/68 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12N C12Q A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	NUCLEIC ACIDS RESEARCH. vol. 20, no. 7 , 11 April 1992 , ARLINGTON, VIRGINIA US pages 1793 - 1799 NORIKO SHIMIZU ET AL. 'Cis-acting elements responsible for muscle-specific expression for the myosin heavy chain beta gene' see abstract see page 1793, right column, paragraph 3 see page 1794, left column, paragraph 4 - right column, paragraph 1 see page 1798, left column, paragraph 2 -paragraph 3 --- -/--	1,3,4,6

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 June 1994	30. 06. 94

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Montero Lopez, B

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 94/00489

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	UCLA SYMPOSIA ON MOLECULAR AND CELLILAR BIOLOGY; NEW SER. vol. 93 , 1989 pages 337 - 347 LEANNE L. CRIBBS ET AL. 'Cis-regulatory elements required for expression of the rabbit myosin heavy chain beta gene' see abstract see page 338, paragraph 3 - page 339, paragraph 2 see page 339, paragraph 5 see page 345, paragraph 1 -paragraph 3 ---	1-4,6,8, 10-12, 14,15
A	JOURNAL OF BIOLOGICAL CHEMISTRY vol. 264, no. 18 , 25 June 1989 , BALTIMORE, MD US pages 10672 - 10678 LEANNE L. CRIBBS ET AL. 'Muscle-specific regulation of a transfected rabbit myosin heavy chain beta gene promoter' see abstract see page 10672, right column, paragraph 3 see page 10673, left column, paragraph 2 -paragraph 3 see page 10673, right column, paragraph 3 see page 10676, right column, paragraph 2 - page 10677, left column, paragraph 1 -----	1,3,4,6

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB94/00489

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark : Although claim 18 is directed to a method of treatment of the human body the search has been carried out and based on the alleged effects of the compound.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.